



**UNIVERSITI PUTRA MALAYSIA**

**GENETIC DIVERSITY AND CHARACTERIZATION OF INDIGENOUS  
RHIZOBIUM LEGUMINOSARUM BIOVAR VICIAE ISOLATES OF  
COOL-SEASON FOOD LEGUMES GROWN IN THE HIGHLANDS OF  
ETHIOPIA**

**NEGASH DEMISSIE TEGEGN.**

**FP 2006 10**

**GENETIC DIVERSITY AND CHARACTERIZATION OF INDIGENOUS  
*Rhizobium leguminosarum* BIOVAR *viciae* ISOLATES OF COOL-SEASON  
FOOD LEGUMES GROWN IN THE HIGHLANDS OF ETHIOPIA**

**By**

**NEGASH DEMISSIE TEGEGN**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,  
in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

**February 2006**



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Doctor of Philosophy

**GENETIC DIVERSITY AND CHARACTERIZATION OF INDIGENOUS  
*Rhizobium leguminosarum* BIOVAR *viciae* ISOLATES OF COOL-  
SEASON FOOD LEGUMES GROWN IN THE HIGHLANDS OF ETHIOPIA**

By

**NEGASH DEMISSIE TEGEGN**

**February 2006**

**Chairman: Associate Professor Halimi Mohd Saud, PhD**

**Faculty: Agriculture**

Cool-season food legumes (CSFLs) are legumes of the temperate cool subtropical origin. In Ethiopian context these legumes encompass 5 legumes such as faba bean (*Vicia faba*), field pea (*Pisum sativum*), lentil (*Lens culinaris*), Chickpea (*Cicer arietinum*) and Grasspea (*Lathyrus sativus*) and are cultivated on the highlands. These legumes have high economic values and provide rich protein sources for human and animal consumption. Although it was reported that, a few species taxonomically related to cultivated CSFL exist in Ethiopian. Thus, there is reason to believe that Ethiopian soils harbour diverse rhizobial isolates, which form symbiotic relationships with CSFLs. In general, there is little or no information is available on the diversity CSFL rhizobia across the country. This indicates that the extent and divergent of the local rhizobial populations belonging to the long cultivated lands are yet to discover. Thus, the importance of characterizing indigenous rhizobia cannot be overemphasized. It is important to establish which rhizobia nodulate which host(s)

and how effectively and which rhizobia predominate which region in order to develop broad host range inoculants in the country. Hence, the current study was conducted with the objectives to isolate, characterise, and determine the morphological, biochemical, and genetic diversity of rhizobial symbionts to CSFLs grown of Ethiopia, and to determine inoculation effects of selected elite strains against introduced/exotic strains on the symbiotic growth and development of lentil (*Lens culinaris*).

Over 150 indigenous *Rhizobium* species, symbionts to CSFLs, were collected from farmers' fields in the highlands of Ethiopia and categorized based on their rhizosphere pH and their agro-ecological zones (AEZ) origin. These isolates were characterised for their colony morphology, host specificity, cell growth rate and mean generation time, acid producing and intrinsic antibiotic resistance (IAR) characteristics. However, a more comprehensive and detail physiological (ATR, STL, IAA, C SUP), and genetic (RAPD, RE RFLP) characterization studies were made apparently for 90 representative isolates of the 150 isolates. Finally, field inoculation experiment was conducted on lentil plant using two elite and 2 exotic inoculant strains. The collection, isolation, colony morphological characterization, and field experiment studies were carried on in Ethiopia, while the physiological and genetic characterization studies were at the Universiti Putra Malaysia (UPM) laboratories in Malaysia.

The study recognized the different rhizosphere pH for the host and existing agro-ecological zones (AEZs) for the initial isolates sampling points and used as a tool to categorize the bulk rhizobial isolates. Thus, isolates constituted 3 and 4 categories for

rhizosphere pH and AEZs, respectively. All isolates were Gram negative *Rhizobium leguminosarum* bv. *viciae* species. Results of the host range specificity study showed that of the 3 rhizobial biovars *Vicia faba* rhizobia were the most host discriminative rhizobia that formed less number of nodules on other host plants. Thus, *Lens culinaris* and *Pisum sativum* rhizobia showed almost similar host discriminative capacity. Isolates differentiated by morphological, acid/alkali production capacity and growth characteristics into 4, 2 and 4 categories, respectively. Approximately 40 % of the total isolates similarly exhibited a colony category of mucoid moisture, with circular shape, white opaque color, and raised structure. However, *Lens culinaris* rhizobia were distinguished apparently by two morphological characteristics. In general, 92% of the indigenous rhizobia isolates examined in this study were fast to very fast growing types acid producing types with overall MGT of 5.88 to 5.9 h.

Eighty-three representative indigenous isolates and 4 reference strains were examined for their physiological characteristics such as ATR, STL, IAA and C SUP and isolates showed variable response. Few rhizobial isolates were able to grow on acid media of pH 4.75 - 6.00. Two *Vicia faba* and one *Pisum sativum* rhizobia were the most acid tolerant isolates that grew at pH 4.75. A total of four *Vicia faba* and two *Pisum sativum* rhizobia were identified to be acid tolerant isolates. In contrary, some isolates of central and northwest highland origins were very sensitive to slightly low pH media of 6. TAL1399 grew apparently on pH 5.5 the reset  $pH > 5.5$ . Some isolates of the same agro-ecology found to have uniform ATR. The response of representative isolates to growth-inhibiting salt (NaCl) concentrations showed relatively low variations among isolates. Most (92%) isolates were able to grow well

at salt concentration of 0.1% and less. The rest 54 % isolates remained unchanged. Almost 78% isolates were tolerated to NaCl upto 0.2%. However, apparently, 3 isolates survived at the highest NaCl concentration of 0.3%. Isolates were homologues with the increased similarity level ( $>0.60$ ) and 17 isolates had shown identical response with that of reference strains. The IAA concentration for the investigated isolates showed that indigenous isolates varied greatly in their IAA production capacity and formed 17 clusters. The IAA concentration reached up to  $25.92 \text{ mg L}^{-1}$  with a mean of  $9.9 \text{ mg L}^{-1}$ . In general, IAA producing capacity of isolates was remained the best indicator to distinguish and group rhizobial isolates. Isolates cluttered into 5 clusters at increased similarity level of  $>0.60$ . IAA was the best indicator among the tested physiological parameters for the divergence of isolates among each other. With respect to C SUP, most isolates preferred polyols, monosaccharide, and disaccharides as their first, second, and third choice carbon sources, respectively. Lentil rhizobia were indifferent for about 6 of the 8 C sources.

Also, results from the current study showed great diversity among isolates with respect to their IAR capacity, making this test useful for distinguishing among isolates. Thirty-three isolates have shown multi-resistant characteristics and formed 18 identical antibiotic resistance profiles. However, number of similar clusters varied with an increase in similarity level of 0.60. Thirteen isolates were found significantly divergent from the bulk of isolates examined. Faba bean and field pea rhizobia had shown more or less uniform IAR capacity, whilst lentil rhizobia showed different IAR capacity for the examined antibiotics.

DNA fragment analysis carried out for 95 representative isolates have shown a total of 83, 79, and 75 fragment patterns for RAPD-PCR, *Hae*III and *Msp*I RE-RFLPs, respectively. These two enzymes per se showed highly polymorphic and distinct DNA profiles indicating the divergence of *Rhizobium* isolates. RE digestion of *Lens culinaris* rhizobia with both enzymes yielded single PCR products with approximately 750 base pairs while the single band for *Vicia faba* and *Pisum sativum* yielded larger fragment of up to 1800 base pairs. Majority (76.17 %) showed significant genetic similarity, while the rest (23.83 %) isolates were divergent among each other. The RAPD-PCR and RE digestions methods formed 18, 13 and 20 clusters respectively. Hence, the DNA profile analysis showed that isolates were distinctly divergent among each other at higher similarity level of >0.60.

Field inoculation experiment on lentil showed that elite inoculant strain EAL400 and imported/ commercial strain TAL1402 in both single as well as mixed inoculant form(s) showed significant ( $P < 0.05$ ) increase in seed yield, yield components, nodulation and symbiotic growth of lentil. The study demonstrated that inoculant type of rhizobial strain sounds better than apparent use of different form(s) (single or mixed inoculants) of inoculants under lentil. Moreover, divergent of rhizobia within *Rhizobium leguminosarum* bv. *viciae* nodulating CSFLs, the field performance of the 2 strains (EAL400 and TAL1402) and the presence indigenous rhizobia that have similar characteristics with strain EAL400 and TAL1402 indicates the future potential for identification of new competitive and efficient *Rhizobium leguminosarum* strains for the country.

Abstrak tesis yang Dikemukakan kepada Senat Universiti Putra Malaysia sebagai  
Memenuhi Keperluan untuk Ijazah Doktor Falsafah

**KEPELBAGAIAN GENETIK DAN PENCIRIAN ISOLAT TEMPATAN  
*Rhizobium leguminosarum* BIOVAR *viciae* YANG BERSIMBIOSIS  
DENGAN DALAM TANAMAN MAKANAN MUSIM SEJUK KEKACANG  
DITANAM DI KAWASAN TANAH TINGGI ETHIOPIA**

Oleh

**NEGASH DEMISSIE TEGEGN**

**February 2006**

**Pengerusi: Professor Madya Halimi Mohd Saud, PhD**

**Fakulti: Pertanian**

Tanaman kekacang musim sejuk (CSFLs) adalah tanaman kekacang yang berasal dari subtropika bercuaca sejuk. Di dalam konteks Ethiopia kekacang ini terdiri daripada 5 jenis kekacang seperti faba bean (*Viciae faba*), field pea (*Pisum sativum*), lentil (dal) (*Lens culinaris*), chickpea (*Cicer arietinum*) dan Graspea (*Lathyrus sativus*) dan ia ditanam di atas tanah tinggi. Kekacang ini mempunyai nilai ekonomi yang tinggi dan membekalkan sumber protein yang kaya untuk kegunaan manusia dan haiwan. Walaupun ia telah dilaporkan bahawa, beberapa spesis secara taksonomik berkait dengan penanaman CSFL yang wujud di Ethiopia. Oleh itu, ia adalah sebab untuk dipercayai bahawa tanah perlindungan Ethiopia mempunyai pelbagai penciran rhizobia, di mana ia membentuk hubungan simbiotik dengan CSFLs. Secara umum, terdapat sedikit atau tiada maklumat yang sedia ada ke atas kepelbagaian rhizobia CSFL merentasi negara. Ini menunjukkan bahawa peluasan dan penyebaran populasi asal rhizobia adalah kepunyaan tanah yang telah ditanam



sekian lama belum lagi ditemui. Oleh itu kepentingan bagi mencirikan rhizobia tempatan tidak boleh terlalu dititikberatkan. Ia adalah penting untuk membuktikan rhizobia yang mana menodulkan perumah dengan berkesan dan rhizobia yang mana mempengaruhi kawasan mana dalam membentuk jumlah inokulan perumah paling banyak dalam negara. Oleh sebab itu, kajian yang sedia ada dijalankan dengan tujuan untuk pencilan, pencirian, dan penentuan morfologi, biokimia, dan kepelbagaian genetik bagi simbiosis rhizobia terhadap CSFLs yang ditanam di Ethiopia, dan untuk menentukan kesan inokulasi bagi strain elit terpilih terhadap strain eksotik ke atas pertumbuhan simbiotik dan pembentukan lentil (*Lens culinaris*).

Lebih 150 *Rhizobium* asli, adalah simbiosis kepada CFLS, telah dikumpulkan daripada ladang petani di tanah tinggi Ethiopia dan dikategorikan berdasarkan pH rhizosfera dan zon agro-ekologi asal. Pencilan ini telah dicirikan terhadap morfologi koloni, perumah spesifik. Kadar pertumbuhan sel dan min generasi masa, penghasilan asid dan ciri rintang terhadap asid. Walaubagaimanapun, kajian yang lebih komprehensif dan fisiologikal lengkap (ATR, STL, IAA, C SUP) dan pencirian genetik (RAPD, RE RFLP) telah dibuat dengan nyata untuk 90 pencilan terpilih daripada 150 pencilan. Akhirnya, eksperimen inokulasi di ladang telah dijalankan terhadap pokok lentil dengan menggunakan 2 strain inokulasi elit dan eksotik. Pengumpulan, pencilan, pencirian morfologi koloni dan kajian eksperimen di ladang telah dilakukan di Ethiopia, manakala kajian fisiologi dan genetik dijalankan di makmal Universiti Putra Malaysia (UPM) di Malaysia. Kajian ini telah mengenalpasti perbezaan rhizosfera bagi perumah dan zon agroekologi (AEZs) yang sedia ada untuk titik permulaan dan digunakan sebagai alat untuk mengkategorikan jumlah keseluruhan pencilan rhizobia. Demikian, secara respektif pencilan mengandungi 3 dan 4 kategori untuk pH rhizosfera dan AEZs. Kesemua pencilan adalah spesis Gram negative

*Rhizobium leguminosorum* bv *viciae*. Keputusan kajian bagi kadar spesifikasi perumah menunjukkan bahawa 3 biovar rhizobia *Vicia faba* adalah rhizobia perumah paling diskriminasi yang membentuk bilangan nodul paling sedikit ke atas pokok perumah yang lain. Demikian, *Lens culinaris* dan *Pisum sativum* menunjukkan kapasiti diskriminasi perumah yang hampir serupa. Pencilan dibezakan melalui morfologi, kapasiti penghasilan asid/alkali dan ciri-ciri pertumbuhan kepada 4, 2 dan 4 kategori secara respektif. Lebih kurang 40% daripada jumlah pencilan menunjukkan persamaan kategori koloni yang berlendir dan lembap, berbentuk bulat, berwarna putih legap, dan struktur tertimbul. Bagaimanapun, rhizobia *Lens culinaris* telah dibezakan melalui dua ciri morfologi. Secara umum, 92% pencilan rhizobia tempatan yang diselidik dalam kajian ini adalah jenis pertumbuhan cepat kepada paling cepat, jenis penghasilan asid dengan jumlah MGT 5.88 ke 5.9 j.

83 wakil pencilan tempatan dan 4 strain rujukan diuji untuk ciri-ciri fizikal seperti ATR, STL, IAA dan C SUP dan pencilan menunjukkan tindak balas yang berubah-ubah. Sesetengah pencilan rhizobia berkebolehan untuk cepat tumbuh di atas media berasid pH 4.75 – 6.00. Dua *Vicia faba* dan satu *Pisum sativum* rhizobia adalah paling rintang terhadap asid dan tumbuh pada pH 4.75. Sejumlah empat *Vicia faba* dan dua *Pisum sativum* rhizobia telah dikenalpastikan pencilan yang rintang terhadap asid. Berlawanan pula dengan sesetengah pencilan asal di tengah dan barat laut kawasan pergunungan yang sangat sensitif kepada media pH serendah 6. Nampaknya TALI399 tumbuh pada pH 5.5 selebihnya pH > 5.5. Sesetengah pencilan yang sama Agro-Ekologi mempunyai ATR yang seragam. Tindak balas wakil pencilan terhadap tahap kepekatan garam yang boleh merencatkan pertumbuhan menunjukkan kepelbagaian di antara pencilan. Kebanyakan (92%) pencilan dapat tumbuh dengan

baik pada kepekatan garam 0.1% dan kurang daripadanya. Selebihnya 54% baki pencilan tidak berubah. Hampir 78% pencilan dapat menahan [NaCl] sehingga 0.2%. Akan tetapi, hanya 3 pencilan yang hidup pada kepekatan NaCl yang tinggi iaitu 0.3%. Pencilan adalah homolog dengan pertambahan persamaan paras ( $>0.60$ ) dan 17 pencilan menunjukkan tindak balas serupa dengan strain rujukan. Kepekatan IAA untuk pencilan yang dikaji menunjukkan variasi tinggi dalam kadar IAA yang dihasilkan dan dapat dikumpulkan dalam 17 kelompok. Kepekatan IAA mencapai lingkungan  $25.92 \text{ mgL}^{-1}$  dengan purata  $9.9 \text{ mgL}^{-1}$ . Secara umumnya, baki pencilan kadar penghasilan IAA merupakan penunjuk yang terbaik dalam membezakan dan mengumpulkan pencilan rhizobia. Taburan pencilan dalam lima kelompok pada tambahan persamaan paras  $>0.60$ . IAA adalah penunjuk terbaik di antara satu sama lain. Dengan pemerhatian kepada C SUP, kebanyakan pencilan memilih polyols, monosakarida dan disakarida pada pilihan pertama, kedua dan ketiga sumber karbon masing-masing. Lentil rhizobia adalah berbeza di antara 6 hingga 8 C sumber.

Sepertimana juga, keputusan daripada kajian semasa menunjukkan kepelbagaian yang besar di antara pencilan dengan hubungan kadar IAR, menyebabkan ujikaji berguna untuk membezakan di antara pencilan. 33 pencilan menunjukkan sifat rintangan-pelbagaian dan menghasilkan 18 profail rintangan antibiotik yang sama. Akan tetapi, bilangan pelbagai kelompok yang sama dengan tambahan di dalam persamaan paras 0.60. Nyata sekali 13 pencilan yang ditemui berbeza di antara sejumlah pencilan yang diuji. Kacang Faba dan rhizobia kacang menunjukkan lebih atau kurang persamaan kadar IAR, sementara lentil rhizobia menunjukkan perbezaan kadar IAR terhadap antibiotik yang diuji.

Analisis fragmen DNA memberikan 95 contoh pencilan yang menunjukkan jumlah 83, 79 dan 75 corak fragmen untuk RAPD-PCR, *Hae*III dan *Msp*I RE-RFLPs, masing-masing. Dua enzim sangat menunjukkan polimorfik dan jelas sekali profail DNA menunjukkan pelbagai pencilan *Rhizobium*. Penguraian RE oleh *Lens culinaris* rhizobia dengan kedua-dua enzim menghasilkan satu produk PCR sekurang-kurangnya 750 bes berpasangan sementara satu jalur untuk *Vicia faba* dan *Pisum sativum* menghasilkan fragmen besar sehingga 1800 bes berpasangan. Majoriti (76.17%) nyata sekali menunjukkan persamaan genetic, sementara selebihnya (28.83%) pencilan berbeza di antara satu sama lain. RAPD-PCR dan kaedah penguraian RE membentuk 18, 13 dan 20 kelompok masing-masing. Dengan itu, analisis profail DNA menunjukkan pencilan tersebut jelas sekali berbeza di antara satu sama lain pada ketinggian persamaan paras 0.60.

Eksperimen penginokulatan ladang ke atas lentil menunjukkan inokulan pencilan elit EAL400 dan strain komersial TAL1402 di dalam keadaan tunggal serta campuran inokulan nyata sekali menunjukkan ( $P < 0.05$ ) tambahan dalam hasil biji benih, hasil-hasil bahan, bintil dan pertumbuhan simbiotik lentil. Kajian membuktikan jenis inokulan tersebut strain rhizobia adalah lebih baik berbanding yang biasa digunakan bagi membezakan bentuk (satu atau campuran inokulan) inokulan di bawah lentil. Lagipun, pelbagai rhizobia dalam lingkungan *Rhizobium leguminosarum* bv. *viciae* bernodulat CSFLs, menunjukkan dua strain (EAL400 dan TAL1402) dan kehadiran rhizobia tempatan mempunyai ciri-ciri persamaan dengan strain EAL400 dan TAL1402 menunjukkan potensi pada masa akan datang untuk mengenalpasti persaingan baru dan keberkesanan strain *Rhizobium leguminosarum* strains kepada negara.

## ACKNOWLEDGEMENTS

First and foremost, I would like to express my warmest, sincerest thanks and deepest gratitude to my supervisors, Associate Professor Dr. Halimi Mohd Saud, Professor Dr. Zulkifli Haji Shamsuddin, and Associate Professor Dr. Raha Abdul Rahim, for their valuable assistance, guidance, knowledge, and encouragement in my study and for the critical review in completion of this thesis. You deserve a special gratitude for believing in me and giving me numerous opportunities particularly in my fieldwork in Ethiopia, insightful advices, and a boost in my scientific endeavours. Special thanks and deepest gratitude to Dr. Asfaw H/Mariam, who assisted, and gave valuable advices and technical support both in rhizobial collection and isolation work. Really, this is the result of your excellent guidance. My appreciation and gratitude goes to the Department of Land Management, Universiti Putra Malaysia (UPM), and the friendly staff, past and present, in Microbiology Section, who facilitated my work at the department. I would like to thank all colleagues I have worked with in the Faculty of Biotechnology and Molecular Sciences of UPM. My warmest gratitude to my colleagues Varma, Yiap, Yanti, Premalatha, Sabrina, Hussien, Elida, and Boon Hee, who shared with me their experiences and showed me wonderful collaboration during my lab work at UPM. It has been great working with these most hard working people I ever met, and many thanks to you all. In addition, I would like to recognize the encouragements made by Prof. Dr. Zaharah Abdul Rahman (UPM), Assoc. Prof. Dr. Siti Zauyah and the current head and coordinator of the soil microbiology laboratory faculty of agriculture, UPM. I would like to thank Dr. Zewdu Terefework of the Department of Applied Chemistry and Microbiology, Division of Microbiology, University of Helsinki, Finland who gave me advices and recommended me important primers, and kindly forwarded valuable information



with regard to molecular analysis. This work was carried out in two phases, the field experiment and rhizobial isolation work back at home in Ethiopia and morphological, physiological and molecular characterization at UPM. I would like to express my appreciation and gratitude to the sponsor of my study Ethiopian government through the Agricultural Research and Training Program (ARTP) of the Ethiopian Agricultural Research Organization (EARO). Of course, my sincerest thanks should go to the administration and management of the Amhara Regional Agricultural and Research Institute (ARARI) and Debre Birhan Agricultural Research Centre (DBRC), centre manager Ato Abebe Mekoya and staff of the Natural Resource Division.

Finally, my family and friends: It would have been impossible for me to accomplish this study without the incredible support and prayer of my family and friends in Ethiopia. I am deeply indebted to my daughter Tsion (Nani) and all my in-laws for their patience and support. Especially all my in-laws you deserve gratitude and great thanks for taking care of my daughter, while both we parents were abroad. I am blessed with friends like you Zewdu Yilma (Dr.) and your wife Alem Bekele, kids Liya, and Surafeal thanks for your unconditional love to my family and me. I hope you will continue to be with me throughout my life. I cannot pass without mentioning your names my friend Tesfaye Chaka, Abera Defabachew and Wondafrash Mulugeta thank you for your assistance in time I needed you. My sisters and relatives Mintiwab Seyifu (Abeba), Hirut, Ayinalem, and my brother Sebsibe, and my mother Elfie, thank you for showing me your unchanged love to me. Thank you, for your encouraging words that kept me going.


I certify that an Examination Committee has met on 17<sup>th</sup> February, 2006 to conduct the final examination of Negash Demissie Tegegn on his Doctor of Philosophy thesis entitled "Genetic Diversity and Characterization of Indigenous *Rhizobium leguminosarum* Biovar *viciae* Isolates of Cool-season Food Legumes Grown in the Highlands of Ethiopia". In accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Member of Examination committee are as follows

**Chek Fauziah, PhD**  
Associate Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Chairman)

**Radziah Othman, PhD**  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Internal Examiner)

**Zainal Abidin Mior Ahmad, PhD**  
Associate Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Internal Examiner)

**Broughton W. J., PhD**  
Professor  
Département de Biologie Végétale  
Faculté des sciences  
Université de Genève  
Switzerland  
(External Examiner)

  
**HASANAH MOHD-GHAZALI, PhD**  
Professor / Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date: **26 APR 2006**

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

**Halimi Mohd Saud, PhD**

Associate Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Chairman)

**Zulkifli Haji Shamsuddin, PhD**

Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Member)

**Raha Abdul Rahim, PhD**

Associate Professor  
Faculty of Biotechnology and Molecular Sciences  
Universiti Putra Malaysia  
(Member)



---

**AINI IDERIS, PhD**

Professor /Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date: 11 MAY 2006



## DECLARATION

I hereby declare that the thesis is based on my original work except for equations and citations, which have been duly acknowledged. I also declare that it has not been previously or currently submitted for any other degree at UPM or any other institutions.



---

NEGASH DEMISSIE TEGEGN

Date 18 APR 2006

## TABLE OF CONTENTS

	Pages
<b>ABSTRACT</b>	iii
<b>ABSTRAK</b>	viii
<b>ACKNOWLEDGEMENTS</b>	xiii
<b>APPROVAL</b>	xv
<b>DECLARATION</b>	xvii
<b>LIST OF TABLES</b>	xxiii
<b>LIST OF FIGURES</b>	xxv
<b>LIST OF PLATES</b>	xxix
<b>LIST OF ABBREVIATIONS</b>	xxx

### CHAPTER

1	<b>INTRODUCTION</b>	1
	1.1 General Objectives	6
	1.2 Specific Objective	6
2	<b>REVIEW OF LITERATURE</b>	7
	2.1 Agroecological Diversity of the Study Area	7
	2.2 Cool-season Food Legumes (CSFLs )	9
	2.3 Microbial Ecology and Rhizosphere	11
	2.3.1 Rhizosphere Colonization	12
	2.3.2 Endophytic Colonization	13
	2.4 The Rhizobia and Natural Microenvironment	16
	2.4.1 Environmental Conditions	16
	2.4.2 Rhizobial Population Dynamics	17
	2.4.3 Acid Tolerance among Rhizobial Species	18
	2.4.4 Salt Tolerance of rhizobia	23
	2.4.5 Soil nutrient	31
	2.5 Biological Nitrogen Fixation (BNF)	34
	2.5.1 Nitrogen Fixing Organisms	34
	2.5.2 The Nitrogen Fixation Process	34
	2.5.3 Actual Contribution of BNF to Agriculture	36
	2.6 Significance of Biological Nitrogen Fixation to Soil Fertility	37
	2.7 Diversity	40
	2.7.1 Rhizobial Diversity	42
	2.7.2 Groupings of Rhizobia and their Separation into Species	44
	2.7.3 Phylogeny	48
	2.8 Symbiosis Between Rhizobia and Legumes	48
	2.8.1 The Rhizobia	48
	2.8.2 Diversity and Specificity of Rhizobium-Legume Association	50
	2.9 Rhizobial Inoculation	54
	2.9.1 Need for Inoculation	54



2.10	Selection of Improved Strains of Root Nodule Bacteria	55
2.10.1	Desirable Characteristics of Root Nodule Bacterial strains	56
2.10.2	Inoculation	56
2.10.3	Mixed Inoculation	58
2.10.4	Incompatible <i>Rhizobium</i> Strains	59
2.10.5	Inconsistent Nodulation and Nodule Effectiveness	60
2.11	Indole-3-Acetic Acid (IAA) Production of Bacteria	61
2.12	Intrinsic Antibiotic Resistance (IAR) among Rhizobia	64
2.13	Phenotypic and Genotypic Assay of Rhizobia	65
2.13.1	Phenotypic Assay	65
2.13.2	Host Plant Assay	66
2.13.3	Biochemical Tests	66
2.13.4	Determining Rhizobia by Numerical Taxonomy	67
2.14	Genotypic Assay	69
2.14.1	Isoenzymes	70
2.14.2	16S rRNA Sequence Analysis	70
2.15	Polymerase Chain Reaction	71
2.15.1	Genetic Fingerprinting by PCR	72
2.15.2	Restriction Fragment Length Polymorphisms (RFLP)	74
2.15.3	Amplified Fragment Length Polymorphism (AFLP)	74
2.15.4	Random Amplified Polymorphic DNA (RAPD)	75
3	<b>GENERAL MATERIALS AND METHODS</b>	77
3.1	Strains Authentication, and Stock Preparation	80
3.2	Storage and Maintenance of Bacterial Strains	81
3.3	Reference Strains	81
3.4	Determination of Genetic Variability of Indigenous Rhizobial Isolates	82
3.5	Statistical Analysis	82
4	<b>COLLECTION, ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF INDIGENOUS RHIZOBIA ISOLATED FROM ROOT NODULES OF COOL-SEASON FOOD LEGUMES (CSFLs) GROWN IN ETHIOPIAN</b>	84
4.1	Introduction	84
4.2	Materials and Methods	86
4.2.1	Geographic Area and Host Crops	86
4.2.2	Rhizosphere pH Characterization	88
4.2.3	Nodule Collection, Rhizobial Isolation, and Identification	89

	4.2.4	Colony Morphology and Gram Reaction	90
	4.2.5	Characterization of rhizobia by growth rates and mean generation times (MGT)	91
	4.2.6	Effect of Isolates on the pH of Growth Media	93
4.3	Results		94
	4.3.1	Geographic Origin and Host Crops of Isolates	94
	4.3.2	Rhizosphere pH, Agroecological Zonation and Representative Isolates	94
	4.3.3	Host Specificity and Effectiveness	100
	4.3.4	Isolation and Identification of Rhizobia	101
	4.3.5	Phenotypic Characteristics of Isolates	102
4.4	Discussion		111
4.5	Conclusion		116
5	<b>PHYSIOLOGICAL CHARACTERISTICS OF</b>	<b>INDIGENOUS <i>Rhizobium leguminosarum</i> BIOVAR <i>viciae</i> ISOLATES OF THE ETHIOPIAN COOL-SEASON FOOD LEGUMES (CSFLs)</b>	<b>118</b>
5.1	Introduction		118
5.2	Materials and Methods		122
	5.2.1	Relative Acid Tolerance Response (ATR) of <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> isolates nodulating Ethiopian CSFLs	122
	5.2.2	Salt Tolerance Level (STL) of <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> Isolates nodulating Ethiopian CSFLs	123
	5.2.3	Indole-3-Acetic Acid (IAA) Production by <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> isolates nodulating Ethiopian CSFLs	124
	5.2.4	Carbon Source Utilization Pattern (C SUP) of <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> Isolates nodulating Ethiopian CSFLs	125
5.3	Results		127
	5.3.1	Relative Acid Tolerance Response (ATR) of <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> isolates nodulating Ethiopian CSFLs	127
	5.3.2	Salt Tolerance Level (STL) of <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> Isolates nodulating Ethiopian CSFLs	132
	5.3.3	Indole-3-Acetic Acid (IAA) Production by <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> isolates nodulating Ethiopian CSFLs	137
	5.3.4	Carbon Source Utilization Pattern (C SUP) of <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> isolates nodulating Ethiopian CSFLs	143
5.4	Discussion		148
5.5	Conclusion		154

6	<b>INTRINSIC ANTIBIOTIC RESISTANCE (IAR) IN INDIGENOUS <i>Rhizobium leguminosarum</i> BIOVAR <i>viciae</i> ISOLATES OF THE ETHIOPIAN COOL-SEASON FOOD LEGUMES (CSFLs)</b>	156
6.1	Introduction	156
6.2	Materials and Methods	157
6.3	Results	159
6.4	Discussion	173
6.5	Conclusion	176
7	<b>GENETIC DIVERSITY OF INDIGENOUS <i>Rhizobium leguminosarum</i> BIOVAR <i>viciae</i> ISOLATES OF THE ETHIOPIA COOL-SEASON FOOD LEGUMES (CSFLs) BASED ON RAPD AND PCR-RFLP METHODS</b>	177
7.1	Introduction	177
7.2	Materials and Methods	180
7.2.1	Bacterial Isolates and Growth Conditions	180
7.2.2	DNA Extraction	180
7.2.3	Phenol-chloroform DNA Extraction Protocol	181
7.2.4	Optimization of PCR Protocol	182
7.2.5	Random Amplification Polymorphic DNA (RAPD) analysis	183
7.2.6	Primers and DNA Markers	184
7.2.7	Electrophoresis	185
7.2.8	PCR-RFLP and Restriction Endonucleases (RE) Digestions Analysis	185
7.2.9	Statistical Analysis	186
7.3	Results	187
7.3.1	Random Amplification Polymorphic DNA (RAPD)	187
7.3.2	PCR-RFLP and Restriction Endonucleas (RE) Digestions	197
7.3.3	Cluster Analysis of Isolates	201
7.4	Discussion	208
7.5	Conclusion	214
8	<b>FIELD INOCULATION EXPERIMENT AND LABORATORY VERIFICATION OF INDIGENOUS ELITE AND EXOTIC RHIZOBIA NODULATING LENTIL (<i>Lens culinaris</i>)</b>	217
8.1	Introduction	217
8.2	Materials and Methods	220
8.2.1	Effect of Selected Elite Inoculant of <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> Strains on Nodulation, Symbiotic Growth, and Yield of Lentil ( <i>Lens culinaris</i> ) under Field Conditions	220

8.2.2	Comparison on the Intrinsic Antibiotic Resistance (IAR) Capacity of Rhizobial Inoculant Strains and Lentil Rhizobia	224
8.2.3	Comparison of the DNA fragment patterns of Rhizobial Inoculant Strains and Lentil Rhizobia using RAPD and RE-RFLP Fingerprintings	225
8.3	Results	226
8.3.1	Effect of Selected Local Elite and Exotic Inoculant Strains on Nodulation, Symbiotic Growth and Yield of Lentil ( <i>Lens culinaris</i> ) under Field Conditions	226
8.3.2	Comparison of the Intrinsic Antibiotic Resistance (IAR) ability of Inoculant rhizobia and re-isolated Lentil rhizobia	242
8.3.3	Comparison of the Genetic Variability of Inoculant Rhizobia and re-isolated Lentil Rhizobia using RAPD and PCR-RFLP RE	244
8.4	Discussion	248
8.5	Conclusion	254
9	<b>GENERAL DISCUSSION AND CONCLUSIONS</b>	256
9.1	Discussion	256
9.2	Conclusions	272
	<b>REFERENCES</b>	278
	<b>APPENDICES</b>	322
	<b>BIODATA OF THE AUTHOR</b>	372

## LIST OF TABLES

Table	Pages
2.1 Traditional Ethiopian agro-ecological zones	8
2.2 Genera and Species of root nodule bacteria of legumes	46
2.3 Characteristics used in the phenotypic and phylogenetic characterization of rhizobia	47
3.1 Host plants, number and source of isolates used in this study	78
3.2 Number of isolates, their initial code and tests performed in this study	79
3.3 Methods used in this study	79
4.1 Broad agro ecological classification of Ethiopia	88
4.2 Nodule samples collected from different localities by host crops and Agro-ecologies of Ethiopia	88
4.3 Summary of the host range test for collected <i>R. leguminosarum</i> bv. <i>viciae</i>	101
4.4 Summary of morphological characteristics of isolates	103
4.5 Number of rhizobial isolates clusters based on their mean growth rate/time (MGT) and rhizosphere pH	106
4.6 MGT calculations made for rhizobial isolates as grouped in their growth rates	108
5.1 Carbon source and concentrations used in this study	125
5.2 Number of indigenous rhizobial isolates and days required to form visible colony development following growth on YEMA, pH 4.5-7.0	128
5.3 Days required for visible colony development of 83 representative rhizobial isolates following growth on agar medium of pH 4.5 – 7.0	136
5.4 Tukey's Studentized Range (HSD) analysis for IAA production capacity of isolates	142
5.5 Number of isolates that showed better C SUP distributed as per isolate species type.	144
6.1 Antibiotics and their concentrations used in this study	158
6.2 Distribution of rhizobial isolates within each IAR cluster (Group I)	161
6.3 Distribution of rhizobial isolates within each IAR cluster (Group II)	164
6.4 Distribution of rhizobial isolates within each IAR cluster (Group III)	166
6.5 Number of rhizobial isolates responded to IAR and their respective host	170
6.6 Strains with identical antibiotics resistance profiles	172

7.1	RAPD-PCR reaction mixtures used for the optimization MgCl <sub>2</sub> :	183
7.2	RAPD-PCR cycling steps and optimized temperature regimes	184
7.3	Sequences of the random oligonucleotide primers tested for RAPD analysis	185
7.4	Characteristics of restriction enzymes used in the study	186
7.5	DNA fragment numbers and size in dominant <i>R. leguminosarum</i> bv. <i>viciae</i> isolates as detected by RAPD-PCR using primer SPH1	189
7.6	DNA fragment numbers of bands produced by each <i>R. leguminosarum</i> bv. <i>viciae</i> isolates per se after digestion with two restriction endonucleases ( <i>Hae</i> III and <i>Msp</i> I).	198
7.7	Number of <i>Rhizobium</i> Isolates Detected by RAPD, RE digestions of <i>Hae</i> III and <i>Msp</i> I	199
7.8	Identical fragment patterns recorded for isolates common for RAPD-PCR, <i>Hae</i> III and <i>Msp</i> I RFLP patterns	201
8.1	Soil physical and chemical characteristics of testing sites	224
8.2	Inoculants used in the field experiments	225
8.3	Influences of different rhizobial strains on shoot height, root length, nodulation pattern seed and yield components of lentil ( <i>Lens culinaris</i> ) at Akaki, Ethiopia	227
8.4	Effect of different rhizobial strains and phosphorus fertilizer on nodulation parameter, seed yield and yield components of lentil at Akaki	229
8.5	Effect of strains and phosphorus on shoot dry mater, nodule number, colour, and fresh weight, (at 50 % flowering stage) and seed protein content at Akaki	230
8.6	Effect of single and combined application of inoculants on root length and dry matter weight, nodules fresh weight and yield and yield components of lentil, at Enewari	234
8.7	Effect of strains and phosphorus on shoot height and dry mater, nodule number, colour and fresh weight, (at 50 % flowering stage) and seed protein content at Enewari	236
8.8	Effect of strains on shoot height, root length, nodulation pattern colour seed yield and yield components at Enewari	237
8.9	Effect of different rhizobial strains on root length and nodulation of lentil ( <i>Lens culinaris</i> ) at Akaki and Enewari sites	238
8.10	Effect of different rhizobial strains on nodules colour and volume of lentil ( <i>Lens culinaris</i> ) at the two sites	240
8.11	DNA fragment numbers and size of lentil <i>rhizobia</i> isolated from lentil inoculation trial at Akaki research station as detected by RAPD-PCR using primer SPH1 and RE digestions	245



## LIST OF FIGURES

Figure	Pages
2.1 Major Agro-ecological Zones (AEZ) of Ethiopia	8
2.2 A conceptual model relating genetic, taxonomic and functional diversity to total biodiversity	41
2.3 <i>Rhizobium</i> legume symbiosis, the partners, the signals, and the response involved in it	52
4.1 Nodules collection areas in Amhara and Oromia regional states	87
4.2 Soil pH Map of Ethiopia	95
4.3 Indigenous isolates collected from root-nodules of different hosts as grouped by the rhizosphere pH and agro-ecological zones of isolates origin.[a] Acid soil pH<6 b] Slightly acidic soil pH 6 – 6.7 and c] Neutral Soil pH 6.8 – 7.2] and agro-ecological origin [M2 – Tepid to cool moist mid highlands; M3 – Cold to very cold sub afro alpine to afro alpine SM2 – Tepid to cool sub moist mid highlands and SM3 – Cold to very cold sub-moist sub-afro alpine]	96
4.4 Percent indigenous rhizobial isolates collected from different legumes as grouped by host plants ( <i>V. faba</i> , <i>P. sativum</i> and <i>L. culinaris</i> and agro-ecological zones of the isolates origin	98
4.5 Number of total and representative rhizobia isolates and their host crop used for this study	99
4.6 Number of isolates per colony morphology group and respective host legume	103
4.7 Rhizobial isolates symbiont to faba bean field pea and lentil based on their growth rate on YMA media	107
4.8 Schematic illustrations of number of rhizobia clustered as very fast, fast, intermediate and slow growing and acid or alkaline producers	110
5.1 Number of rhizobia isolates grown on different pH media as per their host plant and reference strains	131
5.2 Growth (absorbance reading at 600 nm ) for indigenous <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> isolates in comparison with the two reference strains (EAL400 and TAL1402) in different salt concentrations (a-d)	134